

# APPENDIX 1

Sendai virus vector

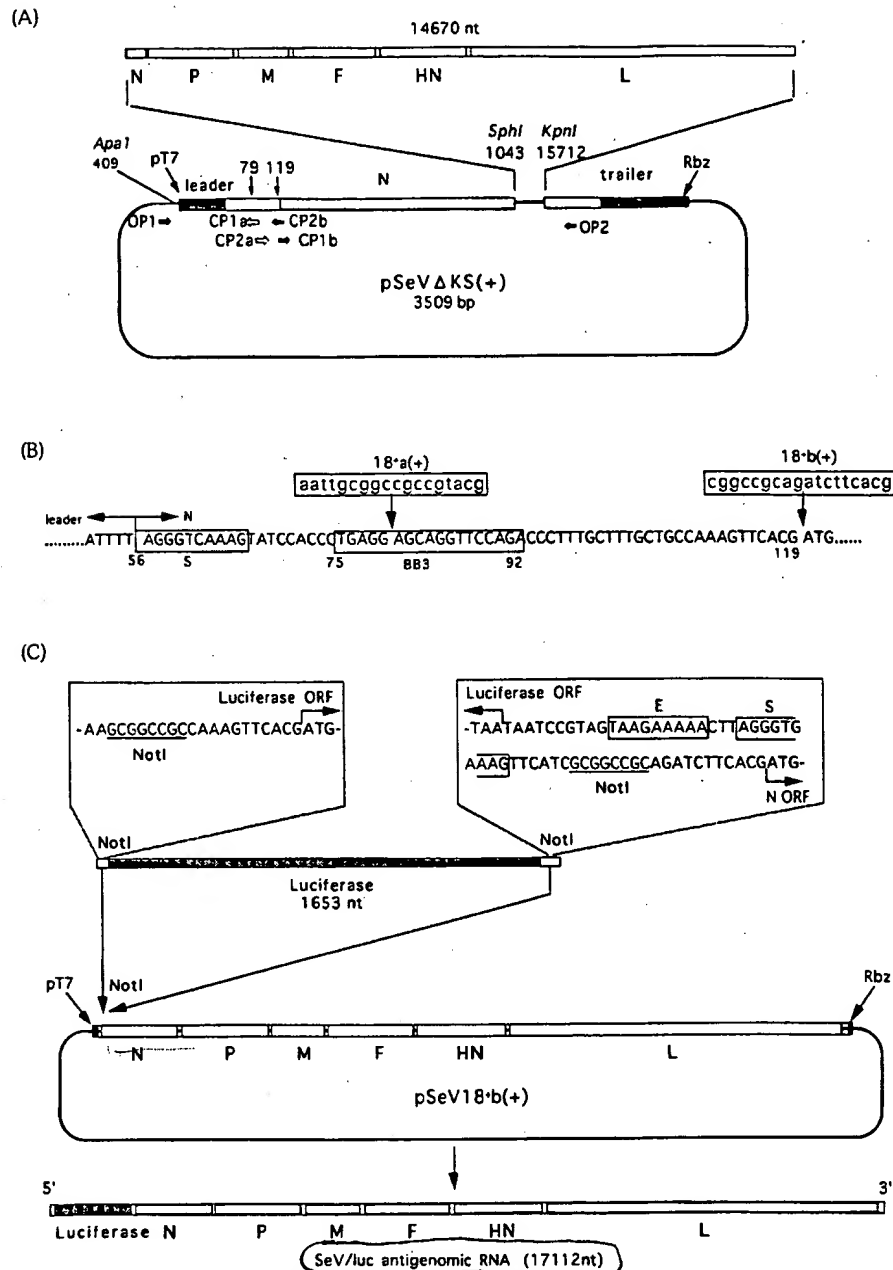


Fig. 1. Construction of the plasmid pSeVluc(+) encoding the SeV antigenome with an insertion of the luciferase gene in the non-coding region of viral N gene. (A) pSeVΔSK was generated by digestion of pSeV(+), encoding the full-length SeV antigenome, with *SphI* and *KpnI* followed by circularization. An 18 nucleotide fragment designed to contain a *NotI* site was inserted after position 79 or 119 by site-directed mutagenesis (PCR-mediated overlap primer extension method; for details, see Methods). CP, complementary primers; OP, outer primers. The larger *SphI*-*KpnI* fragment of SeV spanning the 14 670 nucleotide SeV sequence was then returned back into pSeVΔSK, now containing an extra 18 nucleotides, thereby generating plasmids encoding the SeV antigenome with an additional 18 nucleotide sequence. The plasmid with the insertion after position 119 was named pSeV18<sup>b</sup>(+). (B) The nucleotide sequence (in positive sense) around the site of insertion of the 18 nucleotide fragments [18<sup>a</sup>(+) and 18<sup>b</sup>(+) in lower-case letters]. BB3 is assumed to have critical functions in replication and/or transcription. S represents the start signal for N gene transcription. For details, see the text. (C) The ORF of the luciferase gene was PCR-amplified with *NotI*-tagged primers from plasmid pHvLuc-RT4, digested with *NotI* and introduced into pSeV18<sup>b</sup>(+). A set of new E and S signals connected with GAA was invented in the antisense primer to be placed at the end of the amplified luciferase gene. The resulting plasmid gives rise to an antigenomic SeV RNA of 17 112 nucleotides which contains the luciferase-expressing unit in its first locus.

## APPENDIX 2

*Fields Virology*, Third Edition  
edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al.  
Lippincott - Raven Publishers, Philadelphia © 1996

### CHAPTER 40

# *Paramyxoviridae: The Viruses and Their Replication*

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The viruses of the family Paramyxoviridae are enveloped negative-stranded RNA viruses that have special relationships to two other families of negative-strand RNA viruses, namely the Orthomyxoviridae (for the biological properties of the envelope glycoproteins) and the Rhabdoviridae (for the similarity of organization of the nonsegmented genome and its expression). The genomic RNA of negative strand RNA viruses has to serve two functions: first as a template for synthesis of messenger RNAs (mRNAs) and second as a template for synthesis of the antigenome (+) strand. Negative strand RNA viruses encode and package their own RNA transcriptase, but mRNAs are only synthesized once the virus has been uncoated in the infected cell. Viral replication occurs after synthesis of the mRNAs and requires the continuous synthesis of viral proteins. The

newly synthesized antigenome (+) strand serves as the template for further copies of the (-) strand genomic RNA.

### CLASSIFICATION

The family Paramyxoviridae was reclassified in 1993 by the International Committee on the Taxonomy of Viruses into two subfamilies: the Paramyxovirinae and the Pneumovirinae. The Paramyxovirinae contains three genera, Parainfluenzavirus, Rubulavirus and Morbillivirus. The sub-family Pneumovirinae contains the genus Pneumovirus. The new classification is based on morphological criteria, the organization of the genome, the biological activities of the proteins, and the sequence relationship of the encoded proteins now that most of the genome sequences have been obtained. The morphological distinguishing feature among enveloped viruses for the subfamily Paramyxovirinae is the size and shape of the nucleocapsids (diameter 18 nm, 1  $\mu$ m in length, a pitch of 5.5 nm), which have a left-handed helical symmetry. The biological criteria are (a) anti-

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been observed that is translated to yield a 44-residue protein, SH (114,115). SH is an integral membrane protein that is expressed at the plasma membrane and has the orientation of a type II integral membrane protein (115) (Fig. 8). The functional role of SH in the replicative cycle of SV5 is unknown. For mumps virus, although an mRNA transcript derived from the SH gene has been detected (80,82), attempts to detect a translated protein have as yet been unsuccessful, but the putative mumps virus SH protein does contain a hydrophobic domain sufficient to span a lipid bilayer.

The *Pneumovirus* RS virus encodes a small glycosylated integral membrane protein designated SH (or 1A) (50,52,199). The SH protein contains 64 amino acids and has a single internal hydrophobic domain and sites for the potential addition of carbohydrate at the *N*-terminal region and the *C*-terminal region. By using site-specific antibodies raised to synthetic peptides predicted by the SH gene sequence and immunofluorescent staining techniques, the available data suggest that SH is expressed at the plasma membrane of RS virus-infected cells and that the *N*-terminal domain is cytoplasmic and the *C*-terminal domain extracellular. This membrane orientation of SH as a type II integral membrane protein implies that the *C*-terminal glycosylation site is used. In RS virus-infected cells, four SH-related polypeptide species have been identified: M<sub>r</sub> 4,800, M<sub>r</sub> 7,500, M<sub>r</sub> 13,000 to 15,000, and M<sub>r</sub> 21,000 to 30,000. The M<sub>r</sub> 4,800 species is thought to result from the initiation of protein synthesis at an internal AUG codon; the M<sub>r</sub> 7,500 species is unglycosylated SH; the M<sub>r</sub> 13,000 to 15,000 species is SH containing one high mannose *N*-linked carbohydrate chain; and the M<sub>r</sub> 21,000 to 30,000 species is generated by the addition of polylactosaminoglycan to the *N*-linked carbohydrate chain (5,46,199). The role of the SH protein in the RS virus life cycle is not known.

## STAGES OF REPLICATION

### General Aspects

As far as is known, all aspects of the replication of Paramyxoviridae take place in the cytoplasm. An overview of the life cycle of the virus is shown schematically in Fig. 10, and a schematic diagram indicating the differences between transcription and replication is shown in Fig. 11. Unlike the situation for influenza viruses, Paramyxoviridae mRNA synthesis is insensitive to DNA-intercalating drugs such as actinomycin D (44), and the Paramyxoviridae can replicate in enucleated cells (213). In cell culture, single cycle growth curves are generally of 14 to 20 hr duration, but can be as short as 10 hr for virulent strains of NDV. The effect of viral replication on host macromolecular synthesis is quite variable, ranging from almost complete shut-off late in infection for NDV and Sendai virus to no obvious effect with SV5.

### Virus Adsorption and Entry

For the parainfluenzaviruses and rubulaviruses it has long been accepted that molecules containing sialic acid (sialoglycoconjugates) serve as cell surface receptors. This is based on the fact that sialidase of *Vibrio cholerae* acted as a receptor-destroying enzyme and protected the host cell from infection (172). Sialic acid, the acyl derivative of neuraminic acid, is found on both glycoproteins and on lipids (sialoglycolipids or gangliosides). For Sendai virus it has been shown that gangliosides function as both the attachment factor and the receptor for the virus (171,173,174). As described above, the cellular receptor for the *Morbilivirus* measles virus is the cell surface protein CD46, and the cellular receptor for pneumoviruses is not known. On adsorption of the virus to the cellular receptor, the viral membrane fuses with the cellular plasma membrane at the neutral pH found at the cell surface, the consequence of which is the release into the cytoplasm of the helical nucleocapsids. As discussed above, the attachment protein of many Paramyxovirinae (HN or H) has fusion promoting activity. A model that would rationalize the involvement of HN (H) in the fusion process is one in which the hypothesized conformational change in F to release the fusion peptide is highly regulated. For those Paramyxovirinae that require HN (H) and F to be coexpressed to observe fusion, the first step would be the binding of HN (H) to its receptor. On binding ligand, the HN (H) protein would undergo its own conformational change, which in turn could trigger a conformational change in F to release the fusion peptide. In this way, F and HN operate as a coupled molecular scaffold to release and direct the fusion peptide to the target membrane (157). For viruses such as SV5, for which coexpression of HN only weakly influences cell-cell fusion, the presumptive F conformational change could either be hair-triggered by contact of F with a target membrane or is triggered after docking of F with an unrecognized receptor located on the target membrane (157).

In the virus particle, the M protein is thought to make numerous contacts with the nucleocapsid, and these M-NP contacts are thought to be responsible for inhibiting transcription during virus assembly. On fusion of the viral envelope with the cell plasma membrane and release of the nucleocapsid into the cytoplasm, a mechanism needs to exist to disrupt the M-NP contacts. With influenza A virus, the factor that alters the equilibrium between self-assembly and disassembly is thought to be the difference in pH between the acidic uncoating compartment (endosomes) and the assembly site (plasma membrane). The driving force for paramyxovirus uncoating is not known.

### Primary Transcription

The early evidence indicating that the mRNA of paramyxoviruses was complementary to virion RNA, the

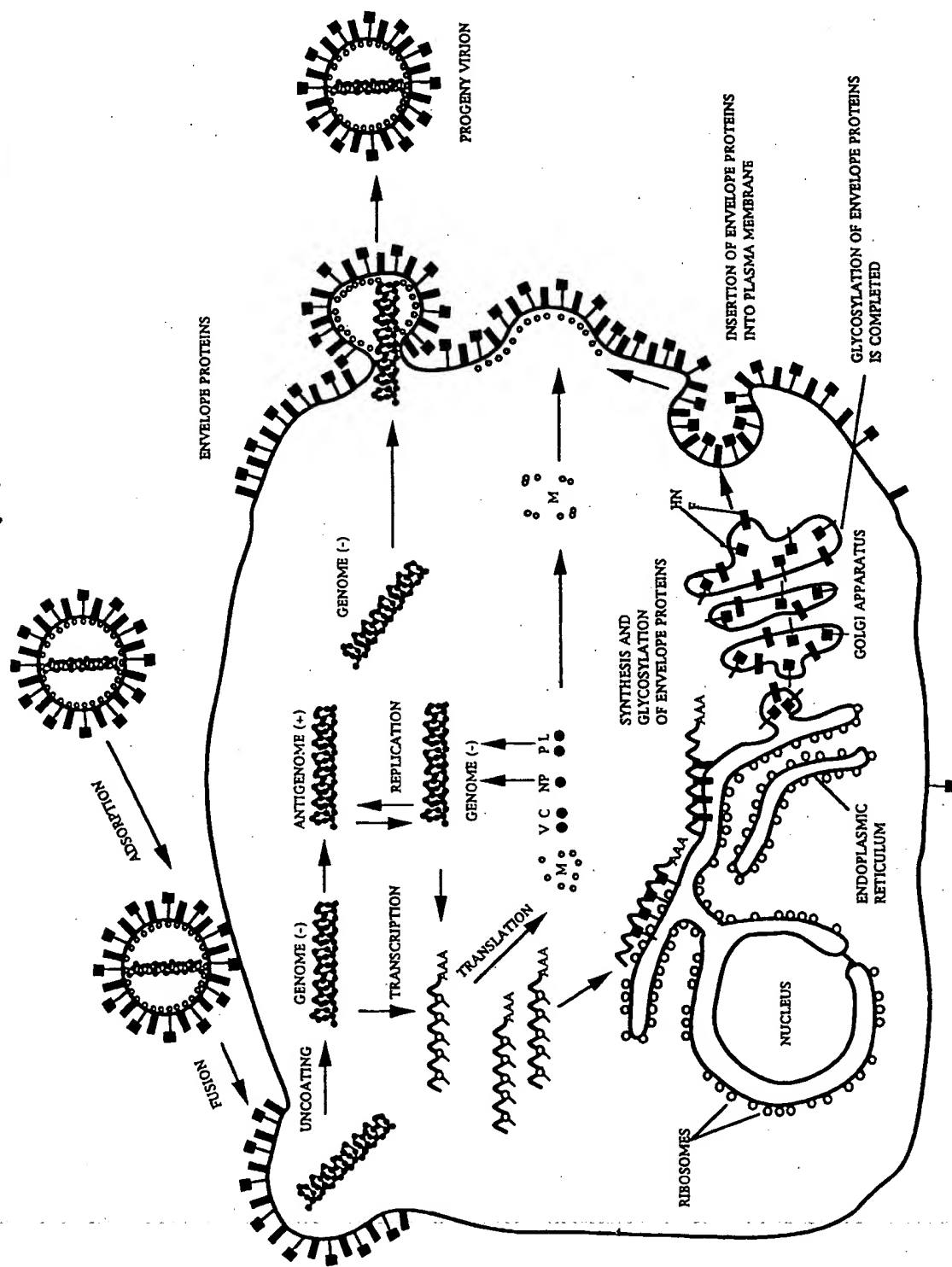
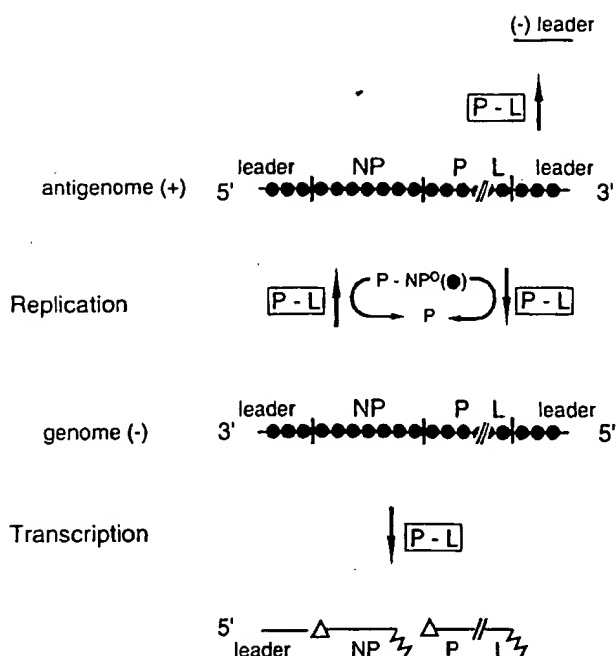


FIG. 10. Schematic representation of the life cycle of a paramyxovirus.



**FIG. 11.** Paramyxovirus RNA synthesis. Viral nucleocapsids, the templates for RNA synthesis, are shown as a linear array of NP subunits (circles), with short vertical lines indicating the gene junctions. The viral polymerase (P-L) transcribes the genome template, starting at its 3' end, to generate the plus leader RNA and the successive capped (triangle) and polyadenylated (squiggly line) mRNAs, by stopping and restarting at each junction. Once these primary transcripts have generated sufficient viral proteins, unassembled NP (as a P-NP<sup>o</sup> complex) begins to assemble the nascent leader chain, and the coordinate assembly and synthesis of the RNA causes the polymerase to ignore the junctions, yielding the antigenome nucleocapsid. In this capacity, P acts as a chaperone to deliver NP<sup>o</sup> to the nascent RNA. The P-L polymerase can also initiate RNA synthesis at the 3' end of the antigenome in the absence of sufficient P-NP<sup>o</sup>; however, only a minus leader RNA is made in this case. Note that genomic and antigenomic RNAs never appear as naked RNAs.

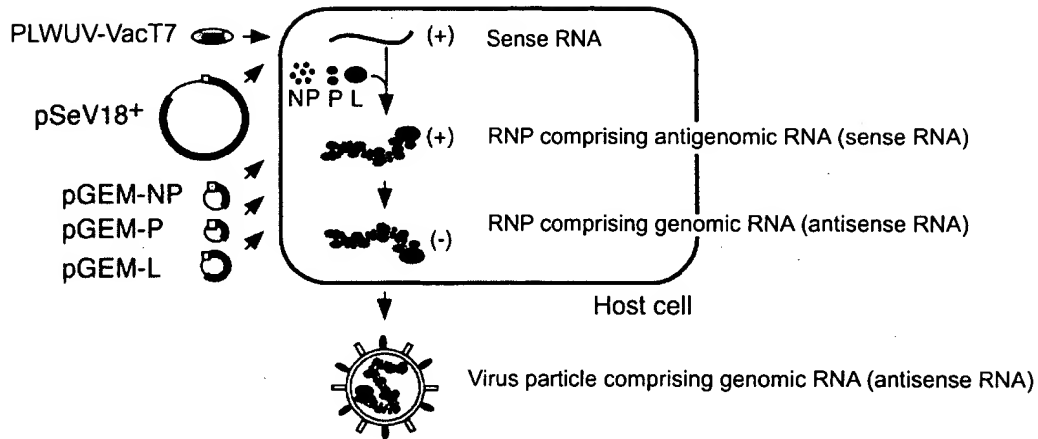
lack of infectivity of naked viral RNA, and the inability to detect RNA-dependent RNA polymerase activity in uninfected cells suggested that a transcriptase might exist in the virions of paramyxoviruses. The first negative strand virus virion-associated polymerase was identified in the Rhabdovirus VSV (7) and shortly thereafter was identified in Newcastle disease virus (129) and Sendai virus (228,262). After fusion of the viral envelope with the host cell plasma membrane has occurred, the infecting nucleocapsids enter the cytoplasm carrying multiple copies of the P/L polymerase. Polymerases are thought to enter the (-) genome template only at its 3' end, but the virion polymerases are scattered over the length of the template (219,220), perhaps frozen in place from when they were shut down for packaging into virions. RNA synthesis is expected to begin as soon as the genome encounters the ribonucleoside triphosphates in the cytoplasm, with the syn-

thesis of the (+) leader RNA. The leader sequences are thought to play a critical role in the control of viral gene expression. The (+) leader sequence separates the 3' end of the genome from the beginning of the first gene, whereas the (-) leader or trailer sequence separates the 3' end of the antigenome from the end of the last gene. These are short sequences, approximately 50 nucleotides long, the first 12 nucleotides of which are identical for the viruses in each genera (and well conserved across the entire subfamily Paramyxovirinae); the remainder of the leader sequence is mostly A and U rich.

In contrast to cellular transcription, in which there are a vast number of genes and the critical choice is often whether to initiate at a given promoter, the promoters of the Paramyxoviridae (or polymerase entry sites) are always "on," and the viral polymerases are restricted to these templates. During primary transcription, the polymerase terminates at the end of the (+) leader region and reinitiates at the beginning of the first gene. Reinitiation appears to be a critical event because it defines a polymerase that transcribes the template to make mRNAs, as opposed to one that replicates the genome. Reinitiation leads to a transcript whose 5' end is capped and whose internal sequences, at specific sites and at specific frequencies, can be cotranscriptionally edited by G insertions. The transcript is also polyadenylated at its 3' end. When the mRNAs of influenza viruses, rhabdoviruses, and paramyxoviruses are aligned on their (-)genome templates, the beginning of the poly(A) tails in all cases line up with a stretch of four to seven U residues on the template. Because polyadenylation of all these viral mRNAs also occurs in polymerase reactions with purified virions, it was suggested that the tails were created by the reiterative copying of the oligo(U) stretch by the polymerase (96,102,104,133,179,226,248). For the *Rhabdovirus* VSV, the polymerase spends as much time crossing the gene junctions as in crossing the much larger coding sequences (133), and virus mutants have been found in which abnormally long poly(A) tails are made (131). Both these findings have reinforced the view that poly(A) addition occurs by polymerase stuttering. More recently, the finding that P gene mRNAs are cotranscriptionally edited by G insertion has suggested that a more controlled form of reiterative copying (or pseudotemplated transcription) also occurs for this process (202,270,276).

The length of the poly(A) tail (approximately 200 residues) is thought to be limited by chain termination. To reinitiate mRNA synthesis at the next start sequence, the polymerase would then skip the intergenic region (which can be as short as a single base or as long as 56 nucleotides for the *Rubulavirus* and pneumoviruses, but is precisely three nucleotides for the *Paramyxovirus* and morbilliviruses). The polymerase would continue transcription in this stop-start fashion until it completed the L mRNA. Thus, except for the short intergenic regions and the trailing (-) leader region, the entire genome is transcribed into mRNA;

### APPENDIX 3: PRODUCTION OF THE RECOMBINANT VIRUS



The plasmid pSeV18<sup>+</sup> encodes an antigenomic RNA (namely sense strand RNA) of the Sendai virus, which is transcribed by T7 RNA polymerase supplied from a vaccinia virus vector PLWUV-VacT7. The plasmids pGEM-NP, pGEM-P, and pGEM-L provide NP, P, and L proteins, respectively.

An antigenomic RNA transcribed from pSeV18<sup>+</sup> forms a ribonucleoprotein (RNP) complex with the NP, P, and L proteins. The RNP comprising the antigenomic RNA, successively generate a genomic RNA (namely antisense strand RNA), which also forms a RNP with NP, P, and L proteins. Only the RNP comprising the genomic RNA is incorporated into the virus particle.

# **APPENDIX 4: COMPARISON OF THE ANTIGENOMIC AND GENOMIC RNAs OF HASAN ET AL. AND THE PRESENT INVENTION**

